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urn:lsid:zoobank.org:pub:CD4009AE-6DB7-49C9-B4FC-B823E8BF5772

Phylogenetic Relationships of the Genus *Homatula* (Cypriniformes: Nemacheilidae), with Special Reference to the Biogeographic History around the Yunnan-Guizhou Plateau

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Abstract

Species of the nemacheilid genus *Homatula* are endemic to the Yunnan-Guizhou Plateau of China. Herein we provide phylogenetic inferences of species relationships based on complete sequences of the mitochondrial *cytochrome b* gene (*cyt b*) and partial sequences of the nuclear *recombination activation* gene (*RAG 1*). Both gene trees (and the concatenated data set) support the recognition of five clades in the genus. These analyses also support the hypothesis that two of the lineages resolved represent undescribed species awaiting formal description, and, further, that two previously recognized species are junior synonyms; both are genetically indistinguishable from other previously recognized species. Divergence times of the major clades in *Homatula* are inferred to be Late Pliocene and the Early Pleistocene, and are correlated with the massive geological events associated with the uplift of the Qinghai-Tibetan Plateau occurring at these times. The reconstruction of drainage histories suggests that the fish faunas of the Nu and Lancang rivers are basal relative to other riverine faunas in the region, and that the Nanpan and the Jinsha rivers share a more recent history than with any other river systems included in our study.

Key words: molecular phylogeny, biogeography, molecular divergence times, Yunnan-Guizhou Plateau

Introduction

Many researchers have argued that biogeographic analyses of extant freshwater fish species can be used to reconstruct historical linkages within and between regional geographical areas and reveal aspects of the underlying diversification processes of biotic evolution (Wiley & Mayden 1985; Mayden 1987). Proposed mechanisms have included dispersal, large- or small-scale vicariance, population divergence through isolation, and genetic drift (Wiley & Mayden 1985; Mayden 1987). Given that freshwater fishes are confined to the lentic and lotic freshwater systems, it has been suggested that their history of evolution and the mechanisms underlying their diversification is probably more easily deciphered than for many other groups (Mayden 1988). Considerable discussion has occurred on the differentiation between divergences via dispersal versus vicariance, but the fact remains that dispersion of primary freshwater fishes depends on the formation of direct connections between drainages (Bermingham & Martin 1998; Lundberg 1993). In recent years our understanding of diversification has been enhanced through phylogenetic inferences coupled with robust estimates of phyletic divergence times. This new approach of combining data sources can provide tremendous insight into our understanding of both the patterns and the underlying the causes of the historical diversification of lineages (Castoe *et al.* 2009).

The Yunnan-Guizhou Plateau is situated at the southeastern fringes of the Qinghai-Tibetan Plateau, shaping the terrace gradient from the Qinghai-Tibetan Plateau to the southeastern flood plain. The Yunnan-Guizhou Plateau is a key area in investigating biotic and environmental responses of species to the geological and geographical

development of this region in concert with the uplift of the Qinghai-Tibetan Plateau. This area is characterized by an extremely rugged terrain made up of a series of high mountains, deeply incised gorges and extremely swift rivers (Ming & Shi 2006). Six major river drainage basins and their associated river systems drain this topographically diverse part of the Yunnan-Guizhou Plateau.

Focused primarily on the amazing landscape of the plateau, most geological and geographical studies of this area have been concerned with the historical development of drainages, primarily relative to the uplift of the Qinghai-Tibetan Plateau, and its impact on other systems (Cheng *et al.* 2001; Clark *et al.* 2004; Ming 2007; Ming *et al.* 2006). Several studies have also used phylogenies of regionally indigenous fishes in concert with geological and geographical information to explain the palaeogeographical histories of the rivers during and following the uplift of the Qinghai-Tibetan Plateau (Guo *et al.* 2005; Peng *et al.* 2006).

The nemacheilid genus *Homatula* (Nichols 1925), as currently defined, is restricted to the eastern slope of the Qinghai-Tibetan Plateau. In the most recent study of the taxonomy of the genus, Gu and Zhang (2011) recognized 12 valid species. *Homatula* was originally described as a subgenus of *Barbatula* by Nichols (1925), based on the type species *Nemacheilus potanini* Günther (1896) from Minjiang (a tributary of the Yangtze River in Sichuan, China). Because species of *Homatula* have an adipose keel developed along the dorsal midline of the caudal peduncle, which strongly resembles that of *Paracobitis* (Bleeker 1863), many researchers have regarded *Homatula* as a synonym of *Paracobitis*, and traditionally ascribed all Chinese balitorid species possessing this keel to the latter genus (Zhu, S.Q. 1989). However, we follow studies by Kottelat (1990), Bănărescu and Nalbant (1995), Hu and Zhang (2010) and Gu and Zhang (2011) and treat species of these two genera as two independent lineages. *Paracobitis* (Bleeker 1863) was described based on the type species *Cobitis malaptera* Cuvier and Valenciennes (1846) from Syria. All species of *Paracobitis* from the western slope of the Qinghai-Tibetan Plateau have 7 branched dorsal-fin rays in the majority of individuals, and a truncate posterior margin of the upper lobe of the caudal fin. By contrast, all species from the Yunnan-Guizhou Plateau, which is situated at the eastern slope of the Qinghai-Tibetan Plateau, have 8 branched dorsal-fin rays in the majority of individuals, and a rounded posterior margin of the upper lobe of caudal fin. In light of the studies cited above, and considering these morphological differences and the large geographic disjunction in the distribution provided by the Qinghai-Tibetan Plateau, we consider these as two separate lineages: *Paracobitis* and *Homatula*. All species endemic to the western slope of the Plateau are *Paracobitis*, and all species endemic to eastern slope of the Plateau are *Homatula*.

The 12 recognized species of *Homatula* are distributed in five major drainages of the Yunnan-Guizhou Plateau: the Nu River (the Salween River Drainage), the Lancang River (the Mekong River Drainage), the Nanpan River (the Pearl River Drainage), the Jinsha River (the Yangtze River Drainage), and the Weihe River (the Yellow River Drainage). Distributions of the species of this lineage thus provide an unusual opportunity to examine the evolution of the headwaters of the major river systems of East Asia. These indigenous fishes are primarily constrained to riverine habitats, although they may occasionally be found in lakes and slowly flowing waters. We felt that a study of their phylogeny and biogeographic relations could provide important insights into the biogeographic effects related to the uplift of the Qinghai-Tibetan Plateau.

Materials and methods

Materials Examined. In order to reconstruct the phylogeny of *Homatula* and to identify related historical biogeographic events, two genes were examined in detail. These were the mitochondrial cytochrome *b* gene (cyt *b*) and the nuclear *recombination activating gene 1* (RAG 1). Tissue samples from 98 individuals of *Homatula* from eighteen localities throughout Yunnan province of China were used in this study (Fig. 1; Table 1). Specimens were identified using the keys and data found in Min *et al.* (2010). Recent phylogenetic studies of Cobitoidea have identified *Schistura* as the sister group of *Homatula*, and *Triplophysa* as the sister group of the *Homatula-Schistura* clade (Tang *et al.* 2006; Šlechtová *et al.* 2007). Eight species of *Schistura* and one species of *Triplophysa* were included in analysis. A total of 107 individuals were examined in this study (Table 1). However, we were constrained by the difficulty in obtaining tissue samples, especially for species recently described or resurrected from synonymy in the eastern portion of the overall range of *Homatula*. Thus, we were unable to include samples of *Homatula nanpanjiangensis* Min, Chen and Yang and *Homatula oligolepis* Cao and Zhu (both Nanpan River Drainage), *Homatula berezowskii* Günther and *Homatula wujiangensis* Ding & Deng (both Yangtze River Drainage), *Homatula laxiclathra* Gu and Zhang (Yellow River Drainage), and *Homatula erhaiensis* Zhu and Cao

(Mekong River drainage). In the cases of the Yangtze and Yellow River species, specimens from the localities in which they occur had previously been identified as *Homatula variegatus* Dabry de Thiersant.

TABLE 1. Species used in this study (KIZ is the abbreviation of Kunming Institute of Zoology).

SPECIES	LOCALITY/ DRAINAGE	VOUCHER NUMBER (DATE)	GENBANK ACCESSION NUMBER	
			CYT <i>B</i>	RAG 1
INGROUP				
<i>H. variegatus</i>	Chuxiong, Yunnan, China / Jinsha River	KIZ 200406167 (2004.6.19)	HM010520	HM010621
		KIZ 200406168 (2004.6.19)	HM010592	HM010623
		KIZ 200406170 (2004.6.19)	HM010573	HM010657
		KIZ 200406171 (2004.6.19)	HM010591	HM010619
		KIZ 200406172 (2004.6.19)	HM010593	HM010620
		KIZ 200406173 (2004.6.19)	HM010587	HM010622
		KIZ 200406174 (2004.6.19)	HM010594	HM010641
		KIZ 200406308 (2004.6.19)	HM010558	HM010640
	Zhaotong, Yunnan, China / Jinsha River	KIZ 200405015 (2004.5.17)	HM010598	HM010638
		KIZ 200405045 (2004.5.17)	HM010590	HM010633
		KIZ 200405046 (2004.5.17)	HM010599	HM010634
		KIZ 200405047 (2004.5.17)	HM010518	HM010639
		KIZ 200405048 (2004.5.17)	HM010589	HM010635
		KIZ 200405049 (2004.5.17)	HM010600	HM010636
		KIZ 200405050 (2004.5.17)	HM010601	HM010637
		<i>H. sp. 2</i>	Heilongtan, Songming, Yunnan / Jinsha River	KIZ 20050114001 (2005.1.14)
KIZ 20050114002 (2005.1.14)	HM010494			HM010630
Muyanghe, Songming, Yunnan / Jinsha River	KIZ 20090051 (2009.11.9)		HM010489	HM010625
	KIZ 20090052 (2009.11.9)		HM010495	HM010624
	KIZ 20090053 (2009.11.9)		HM010492	HM010629
	KIZ 20090054 (2009.11.9)		HM010511	HM010627
	KIZ 20090056 (2009.11.9)		HM010512	HM010628
<i>H. longidorsalis</i>	Yiliang, Yunnan / Nanpan River	KIZ 2008005906 (2008)	HM010500	HM010615
		KIZ 2008005908 (2008)	HM010568	HM010614
		KIZ 2008005909 (2008)	HM010557	HM010617
	Zhanyi, Yunnan / Jinsha River	KIZ 20060274 (2006.12.3)	HM010522	HM010618
		KIZ 20060276 (2006.12.3)	HM010550	HM010616
	Banqiao, Luoping, Yunnan / Nanpan River	KIZ 20080325 (2008.9.20)	HM010579	HM010647
		KIZ 20080326 (2008.9.20)	HM010597	HM010658
		KIZ 20080328 (2008.9.20)	HM010586	HM010645
		KIZ 20080329 (2008.9.20)	HM010596	HM010646
		KIZ 20080330 (2008.9.20)	HM010578	HM010648
	Duoyihe, Luoping, Yunnan / Nanpan River	KIZ 20080331 (2008.9.20)	HM010519	HM010644
		KIZ 20080334 (2008.9.20)	HM010588	HM010643
		KIZ 20080430 (2008.9.23)	HM010575	HM010662
		KIZ 20080431 (2008.9.23)	HM010574	HM010659
		KIZ 20080432 (2008.9.23)	HM010577	HM010660
KIZ 20080433 (2008.9.23)		HM010585	HM010661	

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TABLE 1. (Continued)

SPECIES	LOCALITY/ DRAINAGE	VOUCHER NUMBER (DATE)	GENBANK ACCESSION NUMBER	
			CYT <i>B</i>	RAG 1
<i>H. pycnolepis</i>	Fengqing, Yunnan / Lancang	KIZ 20050331017 (2005.3.31)	HM010541	HM010655
	Gongguoqiao, Yunlong, Yunnan / Lancang River	KIZ 20041001001 (2004.10.1)	HM010523	HM010678
		KIZ 20041003008 (2004.10.3)	HM010521	HM010677
	Jiuzhou, Yunlong, Yunnan / Lancang River	KIZ 20041003009 (2004.10.3)	HM010549	HM010676
		KIZ 05042703 (2005.4.27)	HM010554	HM010656
		KIZ 05043012 (2005.4.30)	HM010555	HM010654
	Lanping, Yunnan / Lancang River	KIZ 20050423003 (2005.4.23)	HM010547	HM010632
	Dahuaqiao, Yunnan / Lancang River	KIZ 05050127 (2005.5.1)	HM010548	HM010652
		KIZ 05050128 (2005.5.1)	HM010559	HM010653
		KIZ 05050129 (2005.5.1)	HM010560	HM010650
	Lincang, Yunnan / Nu River	KIZ 20050421001 (2005.4.21)	HM010567	HM010681
		KIZ 20050421002 (2005.4.21)	HM010553	HM010682
		KIZ 20050421004 (2005.4.21)	HM010552	HM010679
		KIZ 20050421005 (2005.4.21)	HM010501	HM010680
	Longling, Yunnan/ Nu River	KIZ 07308 (2007)	HM010569	HM010663
		KIZ 07310 (2007)	HM010566	HM010683
	Yangbi, Yunnan /Lancang River	KIZ 20100199 (2010)	JN837646	JN837658
		KIZ 20100200 (2010)	JN837647	JN837659
		KIZ 20100201 (2010)	JN837648	JN837660
		KIZ 20100202 (2010)	JN837649	JN837661
		KIZ 20100203 (2010)	JN837650	JN837662
<i>H. anguillioides</i>	Yousuo Spring , Yunnan / Lancang River	KIZ 20080302 (2008.7.22)	HM010546	HM010671
		KIZ 20080303 (2008.7.22)	HM010529	HM010668
		KIZ 20080304 (2008.7.22)	HM010583	HM010669
		KIZ 20080305 (2008.7.22)	HM010582	HM010674
		KIZ 20080306 (2008.7.22)	HM010584	HM010675
		KIZ 20080307 (2008.7.22)	HM010507	HM010664
		KIZ 20080308 (2008.7.22)	HM010506	HM010666
		KIZ 20080311 (2008.7.22)	HM010508	HM010649
		KIZ 20080312 (2008.7.22)	HM010531	HM010667
		KIZ 20080313 (2008.7.22)	HM010581	HM010670
<i>H. acuticephala</i>	Haixihai Lake, Yunnan / Lancang River	KIZ 2008005993 (2008.7)	HM010527	HM010672
		KIZ 2008005994 (2008.7)	HM010503	HM010673
	Haixihai Lake, Yunnan / Lancang River	KIZ 2008005998 (2008.7)	HM010505	HM010665
<i>H. sp. 1</i>	Jiangdong, Wuliang Mountains, Yunnan / Lancang River	KIZ 20080581 (2008.12.13)	HM010517	HM010609
		KIZ 20080582 (2008.12.13)	HM010496	HM010612
		KIZ 20080583 (2008.12.13)	HM010543	HM010611
		KIZ 20080584 (2008.12.13)	HM010532	HM010613
		KIZ 20080587 (2008.12.13)	HM010538	HM010610

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TABLE 1. (Continued)

SPECIES	LOCALITY/ DRAINAGE	VOUCHER NUMBER (DATE)	GENBANK ACCESSION NUMBER	
			CYT <i>B</i>	RAG 1
<i>H. potanini</i>	Jianyang, Sichuan / Jinsha River	KIZ 20080588 (2008.12.13)	HM010535	HM010607
		KIZ 20080591 (2008.12.13)	HM010544	HM010608
		KIZ 2010000231 (2010.1.28)	JF340395	JF340427
		KIZ 2010000232 (2010.1.28)	JF340394	JF340425
		KIZ 2010000233 (2010.1.28)	JF340399	JF340420
		KIZ 2010000234 (2010.1.28)	JF340396	JF340422
		KIZ 2010000235 (2010.1.28)	JF340390	JF340423
		KIZ 2010000236 (2010.1.28)	JF340400	JF340419
	Meishan, Sichuan / Jinsha River	KIZ 2010000237 (2010.1.28)	JF340397	JF340421
		KIZ 2010000238 (2010.1.28)	JF340391	JF340424
		KIZ 2010000239 (2010.1.28)	JF340398	JF340431
		Leshan, Sichuan / Jinsha River	KIZ 2010000266 (2010.1.28)	JF340393
	Jianyang, Sichuan / Jinsha River	KIZ 2010000279 (2010.1.28)	JF340386	JF340434
		KIZ 2010000280 (2010.1.28)	JF340385	JF340426
		KIZ 2010000281 (2010.1.28)	JF340388	JF340388
		KIZ 2010000282 (2010.1.28)	JF340387	JF340432
		KIZ 2010000283 (2010.1.28)	JF340389	JF340428
		KIZ 2010000284 (2010.1.28)	JF340392	JF340429
OUTGROUP				
<i>Schistura fasciolata</i>	Yunnan, China	KIZ 20050320004	HM010513	HM010606
<i>Schistura caudofurca</i>	Yunnan, China	KIZ 200401033	JN837651	JN837663
<i>Schistura callichroma</i>	Yunnan, China	KIZ 200401056	JN837652	JN837664
<i>Schistura amplizona</i>	Yunnan, China	KIZ 2010003103	JN837656	JN837668
<i>Schistura latifasciata</i>	Yunnan, China	KIZ 20050325004	JN837653	JN837665
<i>Schistura cryptofasciata</i>	Yunnan, China	KIZ 20050410011	JF340401	JF340418
<i>Schistura bucculenta</i>	Yunnan, China	KIZ 20080614	JN837654	JN837666
<i>Schistura macrotaenia</i>	Yunnan, China	KIZ 20100035	JN837655	JN837667
<i>Triplophysa stenura</i>	Yunnan, China	KIZ 20040620001	JN837657	JN837669

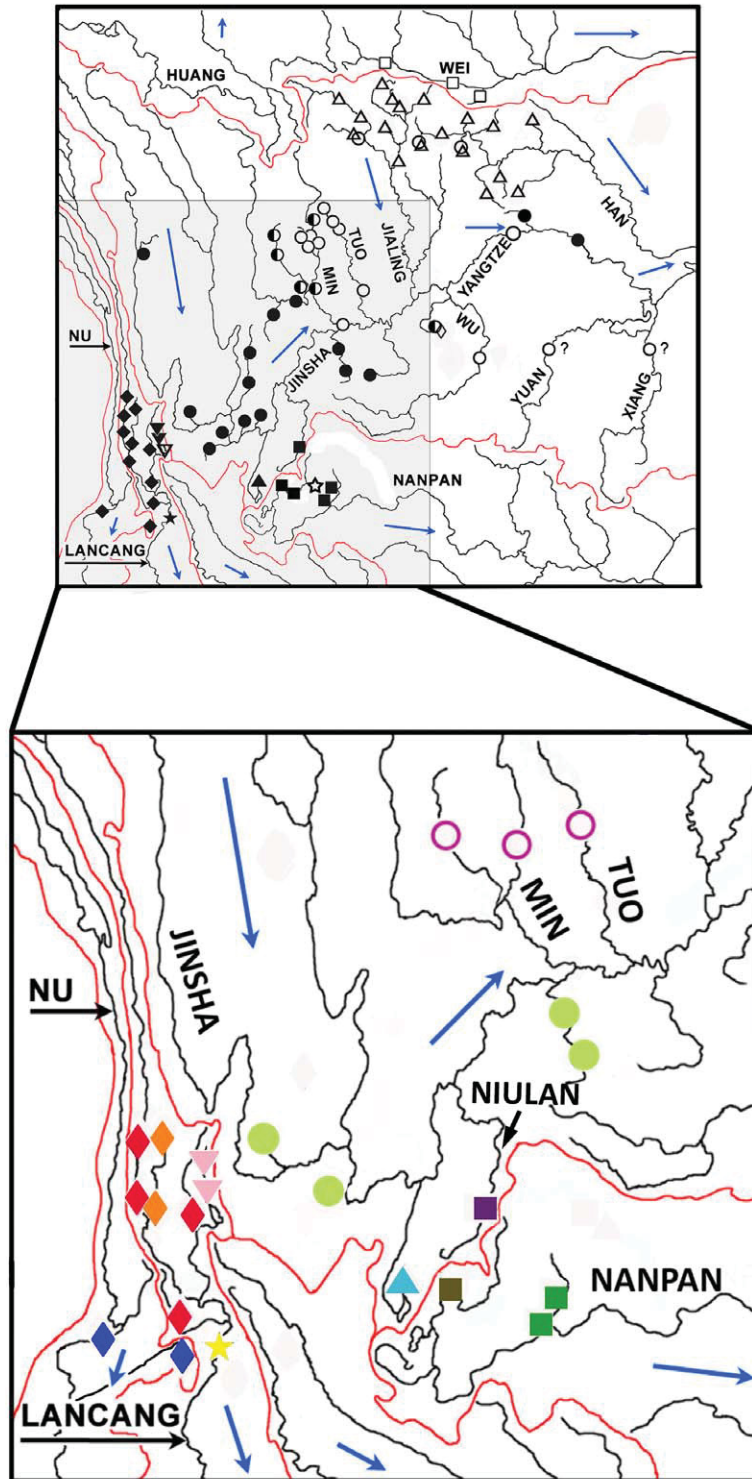


FIGURE 1. Map (above) showing the distributions of *Homatula* based on literature records; map (below) showing the localities of *Homatula* samples used in this study. The area sampled in this study is shaded in the upper map. ◆ *H. pycnolepis*; ▼ *H. acuticephala*; *H. anguillioides*; ★ *H. sp. 1*; ● *H. variegatus*; ■ *H. longidorsalis*; ▲ *H. sp. 2*; ○ *H. potanini*; □ *H. laxiclathra*; △ *H. berezowski*; ◇ *H. wujiangensis*; ▽ *H. erhaiensis*; ☆ *H. nanpanjiangensis*. Different colors represent populations from different locations, and corresponds to the colors used in Figs. 2–5. Blue arrows show the direction of water flow, red lines represent the boundaries of the main drainage basins.

Molecular methods. Total genomic DNA was extracted using a phenol/chloroform technique (Sambrook *et al.* 1989). Primers used to amplify the two genes are listed in Table 2. The PCR reactions were conducted on a T3000 Thermocycler (Biomatra Inc., Germany) in 50 µl volumes containing 38.5 µl of double distilled water, 5 µl of 10× reaction buffer (1% SDS, 50% Glycerol, 0.05% Bromophenol Blue), 3 µl of 2.5 mM dNTP each, 1 µl of 10 µM of each primer, 1 mg of bovine serum albumin, 0.5 µl rTaq DNA polymerase (Takara, Japan) and about 50 ng of DNA template. The following thermal cycling profiles were adopted: 95° predenaturing (5 min), 95° denaturing (40 s), 53° for RAG 1 or 52° for *cyt b* annealing (40 s), 72° extension (90 s), for 35 cycles, and 72° final extension (7 min). PCR products were electrophoresed in 1.5% agarose and recovered using a DNA purification kit (Sangon Inc., Shanghai, China). Sequencing of purified PCR products was performed with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) using the same primers as those used in the PCR. DNA strands were sequenced from both directions.

TABLE 2. Primers used for both PCR amplification and sequencing in present study.

Primer	Primer sequences ^a (5'–3')	Locus	Reference
L14724	GAC TTG AAA AAC CAC CGT TG	<i>cyt b</i>	Xiao <i>et al.</i> 2001
H15915	CTC CGA TCT CCG GAT TAC AAG AC	<i>cyt b</i>	Xiao <i>et al.</i> 2001
RAG-1F	AGC TGT AGT CAG TAY CAC AAR ATG	RAG 1	Perdices <i>et al.</i> 2005
RAGRV1	TCC TGR AAG ATY TTG TAG AA	RAG 1	Šlechtová <i>et al.</i> 2007
R1 2533F	CTGAGCTGCAGTCAGTACCATAAGATGT	RAG 1	López <i>et al.</i> 2004
R14090R	CTGAGTCCTTGTGAGCTTCCATRAAYTT	RAG 1	López <i>et al.</i> 2004

^a Positions with mixed bases are labeled with their IUB codes: R = A/G; Y = C/T.

Sequence data and phylogenetic analyses. Sequences were edited in Seqman and EditSeq program of DNASTar 5 (DNASTAR Inc.) software package, aligned using the alignment explorer in MEGA ver. 4 (Tamura *et al.* 2007), and, if necessary, adjusted by eye. Descriptive statistics for sequence comparisons and chi-square tests of homogeneity of base frequencies across taxa were conducted using PAUP* 4.0b10 (Swofford 2002). Both datasets were tested for saturation by plotting the absolute number of transitions (T_i) and transversions (T_v) against F84 genetic distance in DAMBE (Xia 2000; Xia & Xie 2001). Pairwise distances for two datasets were calculated in MEGA ver. 4.

The Bayesian Inference (BI) of phylogenetic reconstruction was employed using the haplotypes of each marker. Phylogenetic (gene) trees were rooted using *Triplophysa stenura* Herzenstein.

BI analysis was performed using MrBayes ver. 3.1.2 (Ronquist & Huelsenbeck 2003). jModelTest 0.1 (Posada 2008; Guindon & Gascuel 2003) was employed to investigate the evolutionary model that best fit the data set. The Akaike information criteria (AIC) specified TIM3 model + I + G for the *cyt b* gene and the K80 model + G for the RAG 1 gene. For each analysis, two independent Markov chain Monte Carlo (MCMC) processes were run for five million generations with one cold simultaneous chain and three incrementally heated chains, sampling once every 100 generations, and diagnosing once every 1000 generations, with a burn-in of the first 25%. Retained trees were used to construct a 50% majority rule consensus tree indicating the posterior probabilities for nodes.

Divergence time estimation. To infer divergence times within *Homatula*, a likelihood ratio test (Felsenstein 1988) was performed to check whether the constant rate was supported for the *cyt b* data set. Rate constancy in the *cyt b* data set was supported at the $P > 0.05$ level. We used mtDNA for the dating analysis, mainly because it is nonrecombined and the gene provides the most powerful resolution. In the absence of a reliable fossil record for calibration or a local clock-rate estimate for *Homatula*, the relaxed clock option could not be used. Thus, the Bayesian Strict Clock Method for the *cyt b* data set was employed. A Bayesian tree for estimating the divergence time was produced with BEAST 1.61 (Drummond & Rambaut 2007) using the GTR +I+G model, which provided the best fit for this gene. Trees were produced with 10 million MCMC steps with the Yule process under the strict clock with a constant divergence rate 2.65%/Mry (mean divergence rate of 2.5%–2.8%/Mry) obtained for *Lefua echigonia* Jordan and Richardson (Saka *et al.* 2003). A maximum credibility tree was selected using TreeAnnotator 1.61 and visualized with Fig Tree 1.3.1 (A. Rambaut, <http://tree.bio.ed.ac.uk/>).

Results

Sequence characteristics. The sequence information for the specimens examined in this study is listed in Table 1. The 107-taxa *cyt b* data matrix included 1140 aligned nucleotides, of which 416 (36.5%) were variable and 331 (29.0%) were parsimony informative. The 107-taxa RAG 1 data set included 903 aligned nucleotides, of which 149 (16.5%) were variable and 81 (9.0%) were parsimony informative. The sequence characteristics for each codon position are provided in Table 3. For the two datasets, plots of the number of substitutions against F84 distances showed that both T_i and T_v had not reached saturation. The result of pairwise distance for two datasets see Table 4.

TABLE 3. Sequence characteristics of two markers.

Marker	Sites included	Variable sites			Parsimony informative sites (in %)			G	C	T	A
		1 st	2 nd	3 rd	1 st	2 nd	3 rd				
Rag1	903	32	9	108	16(5.32)	4(1.33)	61(20.27)	29.28	26.29	21.88	22.55
Cyt <i>b</i>	1140	78	17	321	56(14.74)	3(0.79)	272(71.58)	15.50	27.27	28.72	28.52

TABLE 4. Pairwise distance for two datasets in *p*-distance.

	Overall	average within <i>Homatula</i>	net between <i>Homatula</i> and <i>Schistura</i>	average between <i>Homatula</i> and outgroups (<i>Triplophysa</i> and <i>Schistura</i>)
Cyt <i>b</i>	0.087	0.067	0.059	0.132
Rag1	0.030	0.020	0.018	0.049

Phylogenetic analyses of protein-coding genes can be biased by the occurrence of homoplasy at the third codon position due to multiple substitutions in transitions (Saitoh *et al.* 2006) and/or due to base composition bias across taxa (Lockhart *et al.* 1994; Chen *et al.* 2003). The χ^2 base composition test was not significant (8.065, d.f. = 318, $P = 1.00000$ for the RAG 1 gene; 47.689, d.f. = 318, $P = 1.00000$ for the *cyt b* gene); the χ^2 base composition test for each 3rd position was not significant (1.446195, d.f. = 318, $P = 1.00000$ for the RAG 1 gene; 170.034, d.f. = 318, $P = 1.00000$ for the *cyt b* gene).

Phylogenetic relationships. Bayesian analyses of the two data sets are fully resolved with respect to the taxa included in this study (Figs. 2 and 3). The following relationships were indicated. The monophyly of the genus *Homatula*, with respect to outgroups, was supported (Bayesian posterior probability (PP) = 1.00). The monophyly of *H. sp. 1* and *H. sp. 2* was supported (PP = 1.00). Five clades were identified in our phylogenetic analysis. Clade I: a basal lineage represented by *H. sp. 1* from a stream in the Wuliang Mountains (flowing into the Lancang River). Clade II: a lineage consisting of *Homatula pycnolepis* Hu and Zhang from the Nu and Lancang Rivers plus *Homatula anguillioides* Zhu and Wang plus *Homatula acuticephala* Zhou and He (see below) from Yousuo, endemic to the Erhai basin (Lancang River drainage). Clade III: a lineage represented by *Homatula potanini* Günther from the Jinsha River. Clade IV: composed of *H. sp. 2* from Songming (Jinsha River drainage). Clade V: contains two sub-clades. One subclade consisted of samples of both *H. variegatus* Dabry de Thiersant (Jinsha River) and *Homatula longidorsalis* Yang, Chen and Kottelat. (Lower Nanpan). The other sub-clade contained only specimens identified as *H. longidorsalis*, but the samples were from both the Jinsha and the Upper Nanpan Rivers.

Conflicts between the gene tree topologies were detected in the following instances: the subclade of *H. anguillioides* plus *H. acuticephala* from Eryuan area and the *H. variegatus*/*H. longidorsalis* group. With the *cyt b* data set (Fig. 2), the subclade of *H. acuticephala* plus *H. anguillioides* from Eryuan area was nested within *H. pycnolepis* from the Nu and Lancang Rivers; the separation of *H. variegatus* and *H. longidorsalis* was not supported. In the RAG 1 gene tree (Fig. 3), the subclade of *H. anguillioides* plus *H. acuticephala* from Eryuan area formed a monophyletic sub-clade with strong support (PP = 1.00) and was the sister group of a monophyletic *H. pycnolepis*. Furthermore, while the monophyly of *H. longidorsalis* was supported with PP of 0.96, that lineage formed a trichotomy with two separate populations of *H. variegatus*.

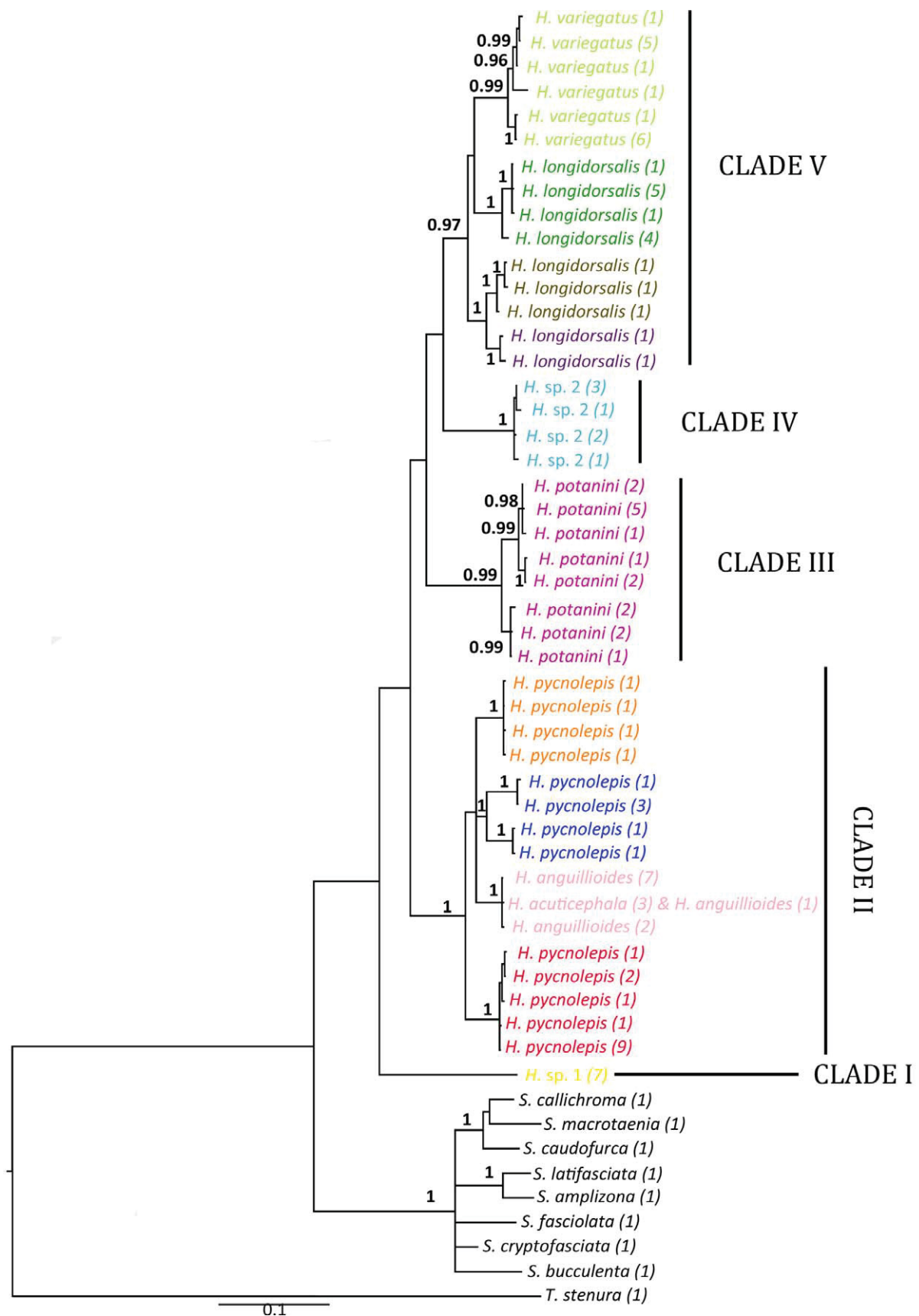


FIGURE 2. 50% major-rule consensus of the Bayesian tree based on the *cyt b* data set with 53 haplotypes. Numbers on node indicate Bayesian posterior probabilities. Numbers in brackets represent the samples of each haplotype. Colors represent population from different locations (see Fig. 1).

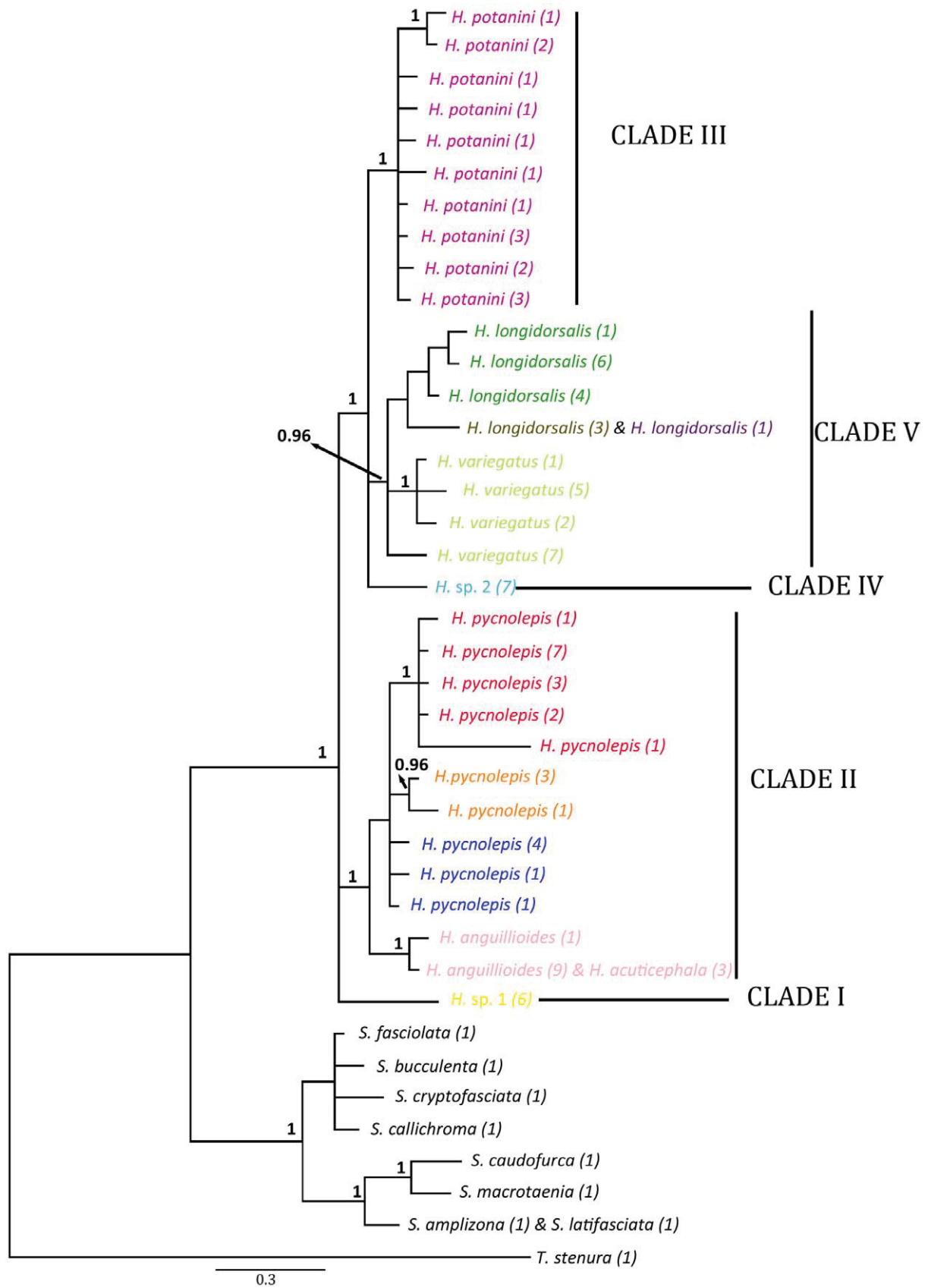
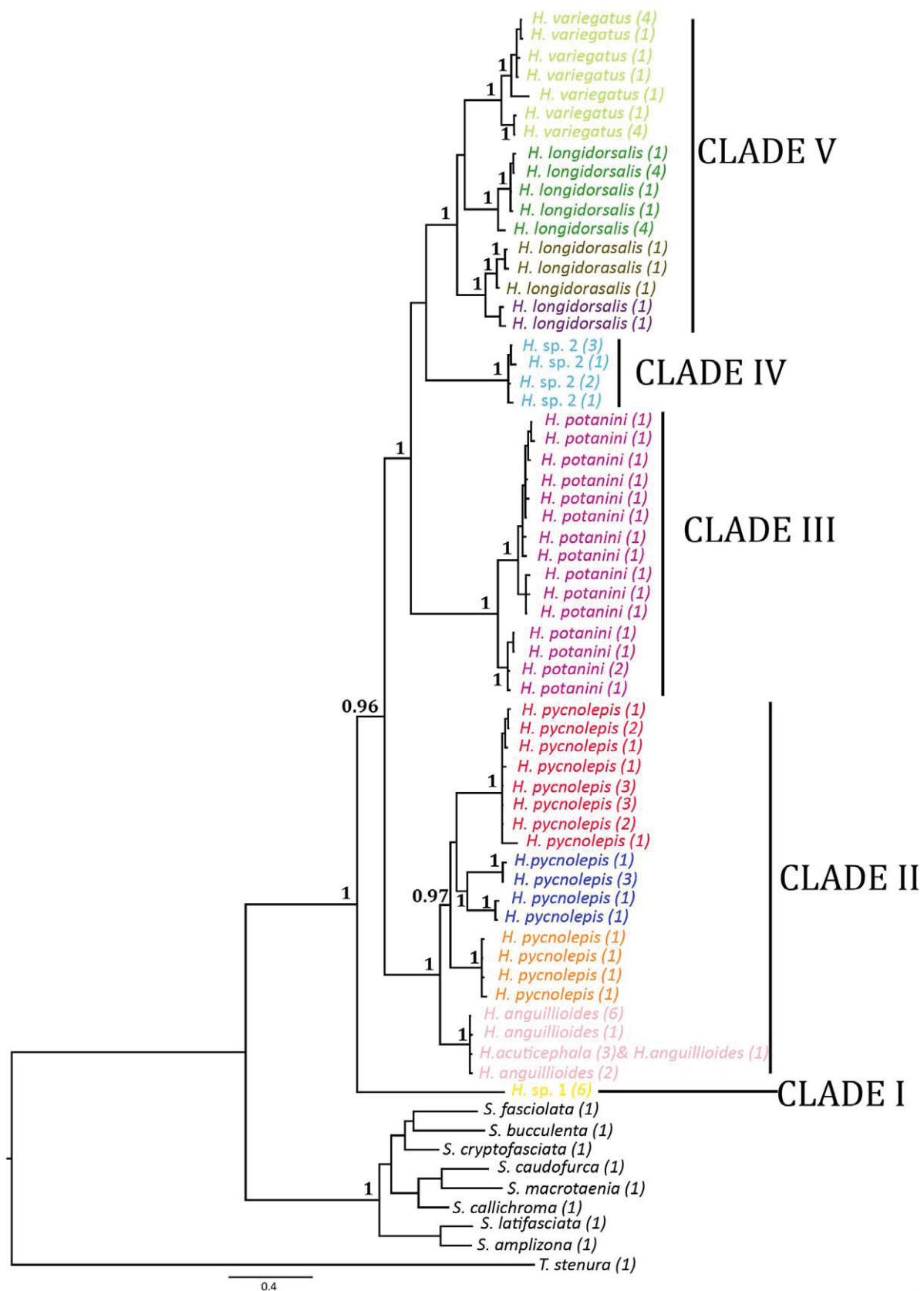


FIGURE 3. 50% major-rule consensus of the Bayesian tree based on the RAG 1 data set with 40 haplotypes. Numbers on node indicate Bayesian posterior probabilities. Numbers in brackets represent the samples of each haplotype. Colors represent population from different locations (see Fig. 1).



a

FIGURE 4. 50% major-rule consensus of the Bayesian tree based on the combined data set with 66 haplotypes. Numbers on node indicate Bayesian posterior probabilities. Numbers in brackets represent the samples of each haplotype. Colors represent population from different locations (see Fig. 1).

Estimation of lineage divergence times. Our dating analyses suggest that *Homatula* originated during the late Pliocene. The major clades were estimated to have diverged during the following times respectively: clade I (*H. sp. 1*) originated around 3.59 Ma; clade II diverged from clade III, IV and V around 2.78 Ma. Within clade III-V, the lineages were estimated to have diverged at the following approximate times: clade III originated around 2.43 Ma; lineage IV diverged from lineage V about 1.95 Ma (Fig. 5).

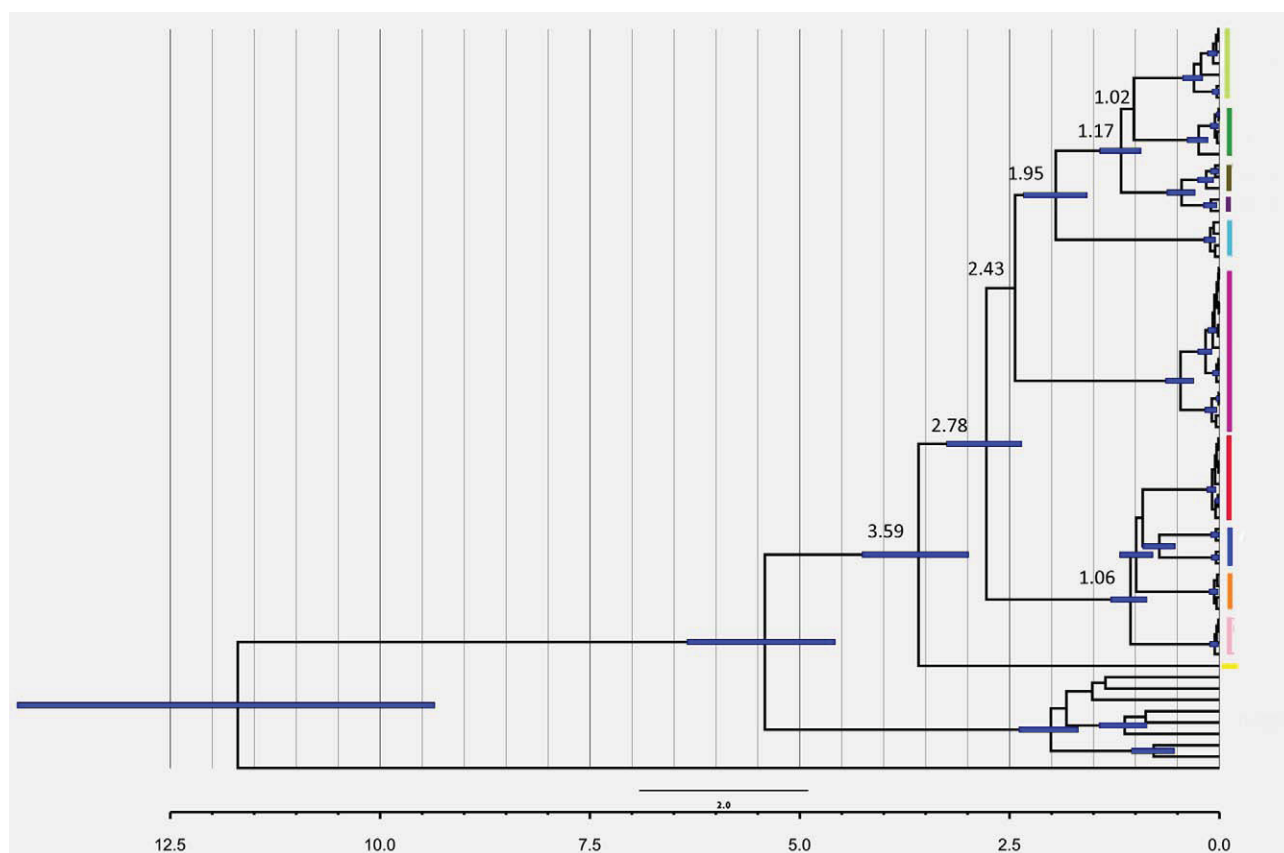


FIGURE 5. Bayesian estimates of divergence time for the *Homatula* phylogeny based on the *cyt b* data set. Bars represent 95% credibility intervals for divergence estimates. Numbers on nodes are million years ago (Ma). Colors represent population from different locations (see Fig. 1).

Discussion

Phylogenetic relationships. Our phylogenetic analyses (Figs. 2 and 3) strongly support the monophyly of *Homatula*, a genus confined to five major drainage basins in China. Since the two trees obtained differed in some details, we used a 50% majority-rule consensus tree derived from the concatenated data set (Fig. 4).

Our analysis indicates that *H. acuticephala* is nested well within the samples of *H. anguillioides* that we examined. *Homatula acuticephala* was described by Zhou and He (1993), and was distinguished from *H. anguillioides* by having a body which is depressed posteriorly from the origin of the dorsal fin (vs. a uniform body depth); a pectoral fin reaching less than half the distance between the origin of the pectoral fin and the origin of the pelvic fin (vs. equal to or slightly less); and 40–42 vertebrae (vs. 40). Our samples from the type locality of *H. acuticephala* (Haixihai Lake) are all juveniles, and the first three putative differences are not apparent. Considering the contradiction between our molecular results and the morphological characters, the status of *H. acuticephala* needs further examination, but we strongly suspect that it will prove to be a junior subjective synonym of *H. anguillioides*. Consequently, we treat all these samples as the latter species in the biogeographic analysis (below).

The distribution of museum specimens originally identified as *H. variegatus* included the upper Yangtze, the Yellow (Wei River) and the Nanpan drainages. However, recent work (Hu & Zhang 2010; Min *et al.* 2010; Gu & Zhang 2011) has shown that a complex of different species is involved. Populations from the Upper Nanpan River (brown symbol on map, Fig. 1) were separated from *H. variegatus* by Yang *et al.* (1994) as a new subspecies, *H.*

variegatus longidorsalis. The major morphological differences cited between two subspecies were the number of branched dorsal-fin rays, the morphology of anterior nostril, the color pattern of head, and the length of maxillary barbels (Yang *et al.* 1994). Although Hu and Zhang (2010) regarded *H. v. longidorsalis* as a junior synonym of *H. variegatus*, Gu and Zhang (2011) resurrected as a full, valid species. Additional collecting since that time has revealed the presence of the *H. longidorsalis* morphotype in a tributary of the Jinsha River (the Niulan Jiang – dark purple symbol, Fig. 1) almost due north of the Upper Nanpan samples. Although the monophyly of *H. variegatus* was supported by our analyses, the monophyly of the two subspecies was not (*cf.* Figs. 2, 3 and 5). In the concatenated tree, *H. variegatus* (lime-green symbol in Fig. 1) forms a monophyletic subunit which is the sister group of the specimens of *H. longidorsalis* from the Lower Nanpan River (dark green symbol in Fig. 1). These two together form the sister group of the specimens identified as *H. longidorsalis* from the Upper Nanpan (the type locality of this species) plus the Niulan branch of the Jinsha. Given the disagreement between the molecular tree and the morphology, it is clear that much further work needs to be undertaken to resolve the phylogeographic relationships among the population of *H. variegatus* throughout its range, and we will treat it further here only at the specific level.

The results of the concatenated data set derived from Cytochrome *b* and RAG 1 can be expressed as (*H. sp. 1* (*H. anguillioides*, *H. pycnolepis*) (*H. potanini* (*H. sp. 2*, *H. variegatus*))).

Biogeographic relationships. Optimization of the area cladogram derived from Fig. 4 suggests the following area relationships (Fig. 6A). The common ancestor of all extant species of *Homatula* occupied the Lancang River. A speciation event occurred within the Lancang system, resulting in the lineage leading to *H. sp. 1* and the ancestor of the lineage that led to all the remaining species. Since the area occupied by the former is extremely small relative to the latter, this is likely to be due to a peripheral isolation mechanism (*sensu* Lynch 1989). The latter ancestor (Clade II, Fig. 4) split into the ancestor of *H. pycnolepis* + *H. anguillioides* (confined to the Lancang and Nu river systems) on the one hand and to the common ancestor of the three remaining species of *Homatula* (presently confined to rivers draining to the east of the Lancang) on the other. The first of the above lineages (Clade II) separated into *H. pycnolepis* (widely distributed in the Nu and Lancang river systems) and *H. anguillioides* (confined to the northern tributary of Erhai Lake). Given the broad distribution of the former relative to the confined range of the latter, the peripheral isolation mode of speciation again seems to be indicated.

It is not possible, on the basis of the present evidence, to decipher whether the physical separation of the lineage leading to *H. potanini*, *H. sp. 2* and *H. variegatus* from the Lancang populations was the result of this speciation event, i.e., whether, in an allopatric model of speciation, the actual speciation event occurred within the Lancang system, or after the separation of the ancestral population into one in the Lancang and the other in the more easterly drainages (which the optimization of current distributions suggests was the Yangtze).

The common ancestor of the three remaining extant lineages then separated into the ancestor of *H. potanini* (currently widely distributed in the Jinsha and its tributaries as well as the Han, and possibly the Yuan and Xiang rivers, all within the Yangtze system) and the common ancestor of *H. sp. 2* and *H. variegatus*. The broad and overlapping distributions of these two lineages are congruent with a hypothesis of sympatric speciation, although allopatric speciation followed by dispersal cannot be ruled out.

The final event was the separation of the ancestor of *H. sp. 2* (confined to the northern tributary stream of Lake Dianchi) and the ancestor of *H. variegatus* (in most of the Jinsha River system). Again, given the disparate sizes of the ranges of these sister species, peripheral isolation is the probable speciation mode.

We recognize that our analysis is incomplete due to the lack of samples, especially from the easterly part of the range. Three of the species recognized by Gu and Zhang (2011) occur in the northeastern streams and rivers of the area (*H. berezowskii*, *H. laxiclathra* and *H. wujiangensis*). However, we hypothesize, based on distribution and overall morphology, that these taxa will be members of the lineage representing Clades III-V, and thus not relevant to the basal biogeographic pattern presented herein. The three other species omitted from the analysis all have very restricted distributions. Two of these are from the Nanpan River system (*H. nanpanjiangensis*, a single locality on the Niujie River) and *H. oligolepis* (Yangzong Lake), and also appear to be members of Clade III-V. The last species unavailable for this analysis is *H. erhaiensis* (Erhai Lake only). Given our conclusion that *H. acuticephala* is a synonym of *H. anguillioides*, and that these two nominal species are only found in a short tributary and lake just to the north of Erhai Lake, it seems highly likely that *H. erhaiensis* is nested within Clade II. Thus, none of these three species would be expected to alter our hypothesized patterns and the basal biogeographic sequence presented here.

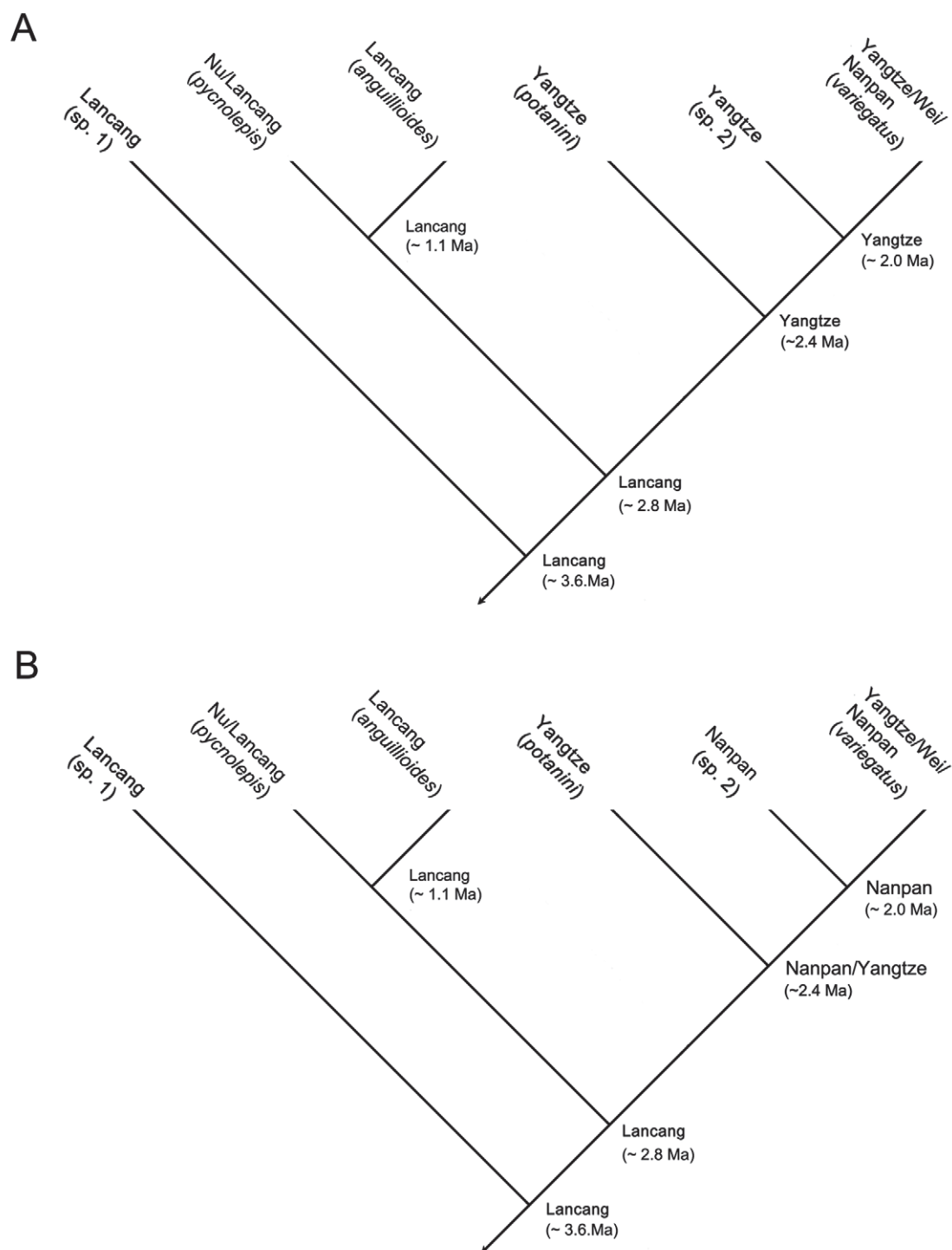


FIGURE 6. Optimization of distributions on an area cladogram. (A) Optimization based on current distributions; (B) Optimization based on incorporation of geological data into current distributions.

Divergence events. The massive Quaternary uplifting of the Qinghai-Tibetan Plateau during the Late Pliocene and the Early Pleistocene resulted from the collision of the Indian and Eurasian Plates. This event, known as the Qingzang Tectonic Movement, took place in three phases (Phases A, B, and C – see Li *et al.* 1996, Shi *et al.* 1999).

The divergence dates of the two initial clades of *Homatula* are congruent with the geological events associated with the uplift of the Qinghai-Tibetan Plateau, which was followed by the formation of the modern drainage

patterns. The time of divergence of the lineage leading to *H. sp. 1*, at around 3.59 Ma (Fig. 5), is very close to Phase A of the Qingzang Tectonic Movement, which has been determined to have occurred around 3.6 Ma (Li *et al.* 2001; Shi *et al.* 1999). This tectonic movement resulted in the breakup of the surface of the plateau and likely of those rivers flowing over this landscape. The rise of the Wuliang Mountain around this time on the southwestern slope of the Yunnan-Guizhou Plateau may well have created a barrier and isolated the common ancestor into two independent lineages: the ancestor of the lineage represented today by *H. sp. 1* and a second ancestral lineage that subsequently evolved into all other species of *Homatula* (Figs. 2, 3 and 4).

Two lineage-splitting events in *Homatula* can be inferred to have occurred at about the time of Phase B of the Qingzang Tectonic Movement (about 2.5 Ma): 1) the divergence of the common ancestor of *H. anguillioides* and *H. pycnolepis* on the one hand and the common ancestor of *H. potanini*, *H. sp. 2* and *H. variegatus* on the other, both dated at about 2.8 Ma, and 2) separation in the lineage giving rise to *H. potanini* from the common ancestor of *H. sp. 2* and *H. variegatus*, dated at about 2.4 Ma (Fig. 5). Phase B of the Qingzang Tectonic Movement was a strong orogenic movement that resulted in the massive uplifting of the Qinghai-Tibetan Plateau to a mean elevation of 1500m to 2000m (Li *et al.* 2001; Shi *et al.* 1999). Since the Yunnan-Guizhou Plateau is situated at the eastern fringe of the Qinghai-Tibetan Plateau, many geologists have proposed that a corresponding contemporaneous uplift movement should have also occurred in this area (e.g. Sun & Zheng 1998). The age we obtained for the separation of the ancestors of *H. anguillioides* + *H. pycnolepis* and the clade *H. potanini*, *H. sp. 2* plus *H. variegatus* (2.8 Ma) might suggest that the isolation of the Lancang River from the Yangtze River occurred around this time. However, the role of geological forces in the separation of the ancestor of *H. potanini* on the one hand and the ancestor of *H. sp. 2* + *H. variegatus* on the other (2.4 Ma) is obfuscated by the extensive sympatry presently existing between *H. potanini* and *H. variegatus*.

During Phase C of the Qingzang Tectonic Movement (1.7 Ma), the Qinghai-Tibetan Plateau was uplifted sharply from a mean elevation of 2000m to 2500m, and is thought to have resulted in the formation of the modern river drainage patterns (Shi *et al.* 1999; Li *et al.* 2001). This date is approximately congruent with the separation of the ancestor of *H. sp. 2* and *H. variegatus* (1.95 Ma). We note that Lake Dianchi formed during the Late Pliocene (pre-2009 definition used here), with a drainage outlet to the Nanpan river system. This drainage was captured by the Jinsha River during the Holocene (within the last 100,000 years, Zhu, H.H. 1989). Given that the date of the speciation of these two species coincides with the formation of Lake Dianchi, the original distribution for *H. sp. 2* must be the Nanpan, not the Jinsha (Yangtze). This interpretation substantially alters the optimization (see Fig. 6B), suggesting that the ancestor of these two species inhabited the Nanpan (not the Yangtze), and that the split between this ancestor and the ancestor of *H. potanini* occurred between the Nanpan and Yangtze, implying that the current occurrence of *H. variegatus* in the Yangtze is secondary.

The most recent speciation event uncovered in our analysis is the separation of *H. pycnolepis* and *H. anguillioides* from their common ancestor some 1.1 Ma. The latter species is confined to the drainage system at the northern margin of Erhai Lake, which formed during the early Pleistocene (pre-2009 definition). The timing of these two events is roughly compatible, and congruent with the formation of the lake initiating speciation.

However, it should be borne in mind that these scenarios are based on a single lineage of six species. Additional phylogenetic trees of additional taxa are needed to evaluate this hypothesis and should be conducted together with time analyses to ensure that divergences are in congruent with the timing and processes outlined here. Whether or not the results we present here are part of a larger biogeographic pattern requiring a general explanation must await additional, larger-scale phylogenetic analyses.

Acknowledgments

We would like to thank all the people who helped with the collection of specimens used in this study, including Li-na Du, Lan-ping Zheng, Jian Yang, Xiao-fu Pan and Wei-ying Wang. We would like to thank Guo-hua Yu, Feng Dong and Kai He for suggestions on data analysis. We also thank Li Jia for help during experiments. This work was funded by the Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX2-YW-Z-0922), the National Natural Science Foundation of China (30730017), Yunnan Provincial Science and Technology Program (2009CC008), and National Science Foundation (USA) Cypriniformes Assembling the Tree of Life (EF 0431326).

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